

The kinetics of ox kidney biliverdin reductase in the pre-steady state

Evidence that the dissociation of bilirubin is the rate-determining step

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When the production of bilirubin by biliverdin reductase was monitored at 460 nm by stopped-flow spectrophotometry a 'burst' was observed with a first-order rate constant at pH 8 of 20 s⁻¹. The steady-state rate was established on completion of the 'burst'. When the reaction was monitored at 401 nm there was no observed steady-state rate, but a diminished pre-steady-state 'burst' reaction was still seen with a rate constant of 22 s⁻¹. We argue that the rate-limiting reaction is the dissociation of bilirubin from an enzyme·NADP⁺·bilirubin complex. With NADPH as the cofactor the hydride-transfer step was shown to exhibit pH-dependence associated with an ionizing group with a pK of 7.2. The kinetics of NADPH binding to the enzyme at pH 7.0 were measured by monitoring the quenching of protein fluorescence on binding the coenzyme.

INTRODUCTION

Biliverdin reductase catalyses the NAD(P)H-dependent reduction of biliverdin to produce bilirubin, which is the major bile pigment in mammalian systems (Colleran & O'Carra, 1977). Bilirubin is recognized as being potentially toxic, so that the conjugation reactions involving glucuronic acid are normally regarded as ensuring the efficient elimination of an undesirable catabolite (Fevry *et al.*, 1972). However, more recent studies have suggested that bilirubin may play an important role as a physiologically important antioxidant (Stocker *et al.*, 1987a,b; Stocker & Ames, 1987). As biliverdin has been suggested to be the major product in at least one of these model systems (Stocker *et al.*, 1987b), it may be that one function of biliverdin reductase is to maintain protective concentrations of a physiologically important antioxidant.

The enzyme exists as a monomer of M_r 34000 in most mammalian species (Noguchi *et al.*, 1979; Kutty & Maines, 1981; Phillips & Mantle, 1981), but has received comparatively little attention. The steady-state kinetics have been studied at pH 9 and shown to follow an ordered mechanism (Rigney & Mantle, 1988). A similar mechanism is probably operative at pH 7.2; however, at this pH the kinetics are not readily amenable to analysis unless a biliverdin-binding protein such as serum albumin is present (see Phillips & Mantle, 1981). The enzyme is subject to potent substrate inhibition as the consequence of the formation of an enzyme·NADP⁺·biliverdin complex (Rigney & Mantle, 1988). The substrate inhibition is partial both at pH 7.2 and at pH 9, as the enzyme·NADP⁺·biliverdin complex can break down via an enzyme·biliverdin complex to form the free enzyme (Phillips & Mantle, 1981; Rigney & Mantle, 1988). We now describe some stopped-flow experiments with the enzyme that provide more detailed information about the mechanism of catalysis. In particular, the observation of a 'burst' of bilirubin formation before the estab-

lishment of the steady-state rate shows that the rate-limiting step of the reaction occurs after hydride transfer.

MATERIALS AND METHODS

Biliverdin reductase was purified from ox kidney as described previously (Rigney *et al.*, 1988). Biliverdin was synthesized by the method of McDonagh (1979). The conditions for studying the steady-state kinetics have been described in Rigney & Mantle (1988), and for details on the pre-steady-state methods, monitoring changes both in absorbance and fluorescence, see Dickinson & Dickenson (1978). Bilirubin formation was generally monitored spectrophotometrically at 460 nm by using $\Delta\epsilon_{460} = 52\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$. For the reverse reaction biliverdin formation was similarly monitored at 660 nm by using $\Delta\epsilon_{660} = 12\,500\text{ M}^{-1}\cdot\text{cm}^{-1}$.

RESULTS AND DISCUSSION

Biliverdin–NADPH reactions

When biliverdin reductase was mixed rapidly with NADPH and biliverdin in 10 mM-glycine/HCl buffer, pH 9.0, containing 0.1 M-Na₂HPO₄ at 25 °C a 'burst' at 460 nm was observed before establishment of the steady-state rate (Fig. 1). Similar experiments were performed in 0.1 M-glycine/HCl buffer at pH 9.0 and with NADH replacing NADPH at pH 8.5 and pH 7.0 in 0.1 M-sodium phosphate buffer. A summary of the results of these experiments appears in Table 1. The characteristics shown in Fig. 1 were also seen in all the other experiments, but some points are worthy of note. Changing from 10 mM-glycine buffer, pH 9.0, containing 0.1 M-Na₂HPO₄ with NADPH as coenzyme diminished the 'burst' amplitude and the apparent first-order rate constant for the 'burst' without materially changing the steady-state rate. No further work has been done on this observation and no specific reason for the changes can be offered. The

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Table 1. Analysis of some stopped-flow experiments with biliverdin reductase at 25 °C

The pre-mixing arrangements were as for Fig. 1. The final concentrations of reactants were 2.7 μ M-enzyme, 100 μ M-NADPH and 20 μ M-biliverdin. The results are expressed as the means and ranges for duplicate experiments.

Buffer	Coenzyme	k_n (s^{-1})	'Burst' amplitude (mol of bilirubin/ mol of enzyme)	Steady-state rate (mol of bilirubin/s per mol of enzyme)
10 mM-Glycine/HCl + 0.1 M- Na_2HPO_4 , pH 9.0	NADPH	12.4 ± 0.2	0.6 ± 0.1	0.62 ± 0.02
0.1 M-Glycine/HCl, pH 9.0	NADPH	5.4 ± 0.1	0.2 ± 0.02	0.69 ± 0.08
0.1 M-Sodium phosphate, pH 8.5	NADH	0.09	0.7 ± 0.1	0.02 ± 0.002
0.1 M-Sodium phosphate, pH 7.0	NADH	5.5 ± 1.0	0.3 ± 0.02	0.35 ± 0.07

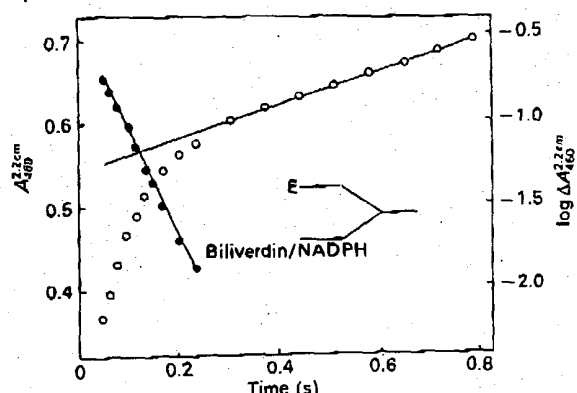


Fig. 1. Pre-steady-state kinetics of biliverdin reductase at pH 9 and 25 °C

Biliverdin reductase was mixed with NADPH and biliverdin to give final concentrations of 3.07 μ M, 50 μ M and 20 μ M respectively, in 0.1 M- Na_2HPO_4 /10 mM-glycine/HCl buffer, pH 9. The reaction curve (O, left-hand scale), the analysis of the transient (●, right-hand scale) and the pre-mixing conditions are shown.

steady-state rates ($v/[E]_0$) observed at pH 9.0 with NADPH as coenzyme are in reasonable agreement with the value of $0.46 s^{-1}$ calculated from eqn. (1):

$$\frac{v}{[E]_0} = \frac{k_{cat}[A][B]}{K_m + K_m[A] + K_m[B] + [A][B]} \quad (1)$$

by using the steady-state parameters determined earlier (Rigney & Mantle, 1988). [In eqn. (1) A and B represent NADPH and biliverdin respectively.] At alkaline pH NADH is clearly a very poor substitute for NADPH with this enzyme. Both the 'burst' rate constant and the steady-state rate are much smaller, although the 'burst' amplitude is similar to that for NADPH. In previous experiments with much smaller concentrations of enzyme we were not able to observe any activity with NADH at pH 8.5 (Rigney & Mantle, 1988). It is clear now that NADH is an effective coenzyme at pH 7.0 and that the broad features of the reaction mechanism are the same as for NADPH.

Stopped-flow experiments with the same pre-mixing arrangements as for Fig. 1 were continued at pH 7.0 in

0.1 M-phosphate buffer. NADPH was the coenzyme. As is shown below, the 'burst' rate constant is much higher than at pH 9.0, although the steady-state rates and 'burst' amplitudes are similar at the two pH values. Variation of the biliverdin concentration (5–80 μ M) with 100 μ M-NADPH and variation of the NADPH concentration (100–1100 μ M) with 5 μ M-biliverdin established that the 'burst' rate constant ($135 \pm 15 s^{-1}$), the 'burst' amplitude (0.45 ± 0.5 mol of bilirubin/mol of enzyme) and the specific steady-state rate ($1.2 \pm 0.15 s^{-1}$) remained essentially constant over the concentration ranges tested. Thus the characteristics observed at pH 9.0 (Fig. 1) are also seen at pH 7.0, and it appears that the concentrations used for Fig. 1 and for the experiments described below are saturating.

The effect of pH on the behaviour of the enzyme was examined in a series of experiments with NADPH as coenzyme. The important results appear in Table 2 and Fig. 2. The 'burst' rate constant (k_n) is strongly pH-dependent with an apparent pK_n of 7.2. A finite value of $k_n = 10 s^{-1}$ seems to be achieved at alkaline pH, suggesting that protonation of this group may not be

Table 2. pH variation of the reaction profiles for biliverdin reductase at 25 °C with NADPH as coenzyme

Experiments were conducted in 0.1 M-sodium phosphate buffers with the pre-mixing arrangement shown in Fig. 1. At pH 9.0 the buffer was 10 mM-glycine/HCl containing 0.1 M- Na_2HPO_4 . The final concentrations of reactants were 2.7 μ M-enzyme, 100 μ M-NADPH and 20 μ M-biliverdin. The results are expressed as the means and ranges for duplicate experiments.

pH	k_n (s^{-1})	'Burst' amplitude (mol of bilirubin/ mol of enzyme)	Steady-state rate (mol of bilirubin/s per mol of enzyme)
6.0	162	0.35	1.0
6.5	171 ± 5	0.35 ± 0.02	1.1 ± 0.1
7.0	136 ± 7	0.43 ± 0.02	0.68 ± 0.05
7.25	97	0.44	0.61
7.5	53 ± 5	0.6 ± 0.04	0.72 ± 0.07
8.0	20 ± 1.0	0.63 ± 0.01	0.59 ± 0.1
8.5	13 ± 0.5	0.5 ± 0.01	0.68 ± 0.02
9.0	12 ± 1	0.58 ± 0.05	0.58 ± 0.01

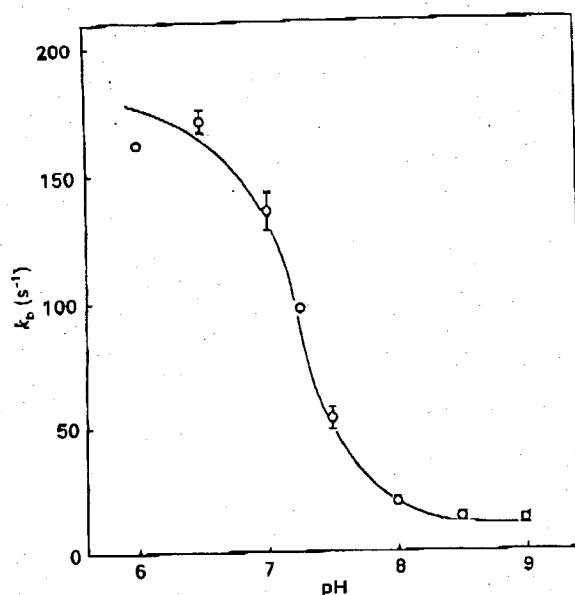


Fig. 2. Effect of pH on the 'burst' rate constant of biliverdin reductase at 25 °C

A solution containing 6.14 μM -biliverdin reductase in 2 mM-sodium phosphate buffer, pH 7.2, was mixed with a solution containing 40 μM -biliverdin and 100 μM -NADPH in the following buffers: 200 mM-sodium phosphate, pH 6–8.5; 200 mM-sodium phosphate/20 mM-glycine, pH 9. The symbols and bars show the means and ranges for duplicate estimates.

absolutely critical for the reaction to proceed with NADPH as coenzyme. The same may not be true for NADH. The limited data of Table 1 show that k_b for NADH changes in the same direction as for NADPH, but much more dramatically between pH 7.0 and pH 8.5. Comparison of the steady-state rates (Tables 1 and 2) with NADPH and NADH also shows a marked difference between the two coenzyme reactions. For NADPH the steady-state rate is rather insensitive to pH, whereas for NADH the rate at pH 7.0 is comparable with that for NADPH but at pH 9.0 is extremely slow. In view of these differences it is interesting that the 'burst' amplitudes for the two coenzymes are quite similar and are relatively pH-independent.

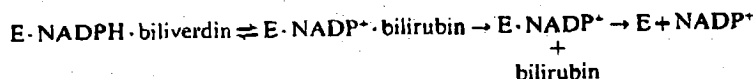
The stopped-flow experiments indicate that the rate-limiting step in biliverdin reduction occurs after hydride transfer and bilirubin formation. A likely mechanism to explain the results is shown in Scheme 1, which describes the events following mixing of enzyme with saturating concentrations of biliverdin and NADPH. In this simple scheme two possible rate-limiting steps occur after

hydride transfer, and it is not uncommon to find within the class of NAD(P)-linked dehydrogenases that product coenzyme dissociation is the rate-limiting reaction. In the present case, however, it does not seem that this is so. The 'burst' amplitudes are in all cases significantly less than the 1.0 mol/mol of enzyme expected if NADP⁺ dissociation is the only rate-limiting step. The low values could be explained if the enzyme preparation was only partially active, but as these preparations were highly active and appear to be homogeneous by various criteria (Rigney & Mantle, 1988) this possibility is for the present discounted. It is also possible that the absorption coefficient of enzyme-bound bilirubin at 460 nm is significantly lowered from that of bilirubin in solution. It seems unlikely on general grounds, however, that the absorption coefficient would be affected by as large a hypochromic shift as would be necessary to be compatible with 'burst' amplitudes of 1.0 mol/mol of enzyme. Certainly the experiments described below at different wavelengths suggest only a limited effect on the absorption spectrum of bound bilirubin.

The data presented can be perhaps best explained by assuming that the rate-limiting step in the reaction is bilirubin dissociation from the product enzyme·NADP⁺·bilirubin complex. The hydride-transfer step would rapidly equilibrate at a rate determined partly by the state of ionization of the enzyme group having pK_a 7.2, but the equilibrium position of the hydride-transfer step may be roughly in balance. As the bilirubin then dissociates the hydride-transfer step re-equilibrates to maintain the balance and the enzyme·NADPH·biliverdin complex concentration is restored rapidly by combination of substrates with free enzyme liberated by the dissociation of NADP⁺. With yeast alcohol dehydrogenase, for example, the equilibrium constant for the hydride-transfer step is about 0.1 at pH 7.0 (Dickenson & Dickinson, 1978) whereas the equilibrium constant for the overall reaction is 10^{-4} at this pH (Bäcklin, 1958).

An argument in favour of the above proposal is that in all cases the transient seen in stopped-flow studies is strictly first-order over at least 90% of the reaction course. There is no evidence either in the experiments described above or in those given below that two separate kinetic processes contribute to the appearance of bilirubin in the pre-steady state. If NADP⁺ dissociation from the terminal enzyme·NADP⁺ complex is rate-limiting and the hydride-transfer step is rapid and roughly balanced at equilibrium, one would expect to see a biphasic course of bilirubin formation in the pre-steady state whether or not bilirubin and the enzyme·NADP⁺·bilirubin complex have the same absorption coefficient.

A series of experiments like that described in Fig. 1 were conducted at pH 8.0 with observations being made in the wavelength range 393–413 nm. The results are



Scheme 1. Proposed mechanism for biliverdin reductase

The mechanism is a shortened form of a compulsory-order mechanism with coenzyme as the leading substrate. This form of the mechanism is applicable to the stopped-flow experiments described in the text where saturating concentrations of NADPH and biliverdin were used.

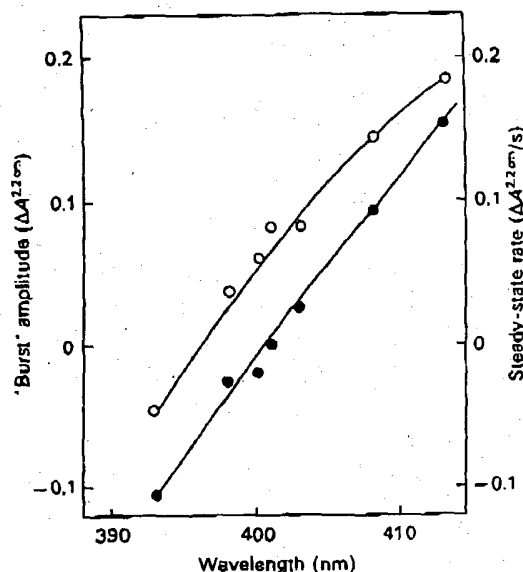


Fig. 3. Wavelength-dependence of 'burst' amplitude and steady-state rate of biliverdin reductase at pH 8.0 and 25 °C

Biliverdin reductase (10.06 μM) was mixed with NADPH (100 μM) and biliverdin (20 μM) in 0.1 M-sodium phosphate buffer, pH 8.0, in the stopped-flow apparatus. The reactions were monitored at various wavelengths and the 'burst' amplitude (\circ , left-hand scale) and steady-state rate (\bullet , right-hand scale) were measured.

shown in Fig. 3. It is clear that the 'burst' amplitude decreases as the wavelength decreases. There is an isosbestic point at about 396 nm when no 'burst' is observed, and thereafter the sign of the 'burst' is inverted. The steady-state rate shows the same trend as the 'burst', but the curve is shifted to longer wavelengths and the isosbestic point is at 401 nm. These experiments show that the absorption spectrum of bilirubin in the enzyme·NADPH·bilirubin complex is shifted to shorter wavelengths from that of bilirubin in free solution. The shift is not very great (approx. 4 nm), however, and as the absorption band is rather broad (approx. 90 nm at half maximum) it is unlikely, as stated above, that the absorption coefficient of bound bilirubin at 460 nm is greatly lowered from that of bilirubin in solution.

The transients documented in Fig. 3 are all strictly first-order with experimental error regardless of the sign of the 'burst' and all give values for the 'burst' rate constant in the range of $k_b = 20 \pm 2 \text{ s}^{-1}$. Obviously in the region of the isosbestic point the accuracy of the determinations is decreased because of the diminished magnitude of the transient. The fact that the pre-steady-state kinetics of bilirubin formation remain simple across a wide wavelength range supports the view that only one species of enzyme-bound bilirubin is kinetically significant. This encourages belief in the interpretation of the pre-steady-state experiments observed above.

Bilirubin-NADP⁺ reactions

A few stopped-flow experiments were conducted at 25 °C in 0.1 M-sodium phosphate buffer, pH 8.5, in which enzyme was rapidly mixed with a solution of bilirubin

and NADP⁺. The final concentrations after mixing were 2.7 μM -enzyme, 80 μM -bilirubin and 1 mM-NADP⁺. The reactions were monitored spectrophotometrically at 660 nm. The reaction profiles were very similar to that of Fig. 1 in that a rapid 'burst' of absorbance was followed by establishment of a slow steady-state rate. In these experiments the 'burst' amplitude was 0.15 ± 0.02 mol of biliverdin/mol of enzyme, the 'burst' rate constant k_b was $5.2 \pm 0.5 \text{ s}^{-1}$ and the steady-state rate was 0.02 ± 0.003 mol of biliverdin/s per mol of enzyme. It was not established whether the concentrations of substrates were saturating, but it seems likely that they were.

As for the forward reaction the rate-limiting step in bilirubin oxidation seems to occur after hydride transfer. The hydride-transfer step may equilibrate rapidly, with the equilibrium position being in favour of the enzyme·NADP⁺·bilirubin complex. It is possible that the steady-state rates observed are inhibited rates because of the steady formation of the abortive enzyme·NADP⁺·biliverdin complex (Rigney & Mantle, 1988). Accordingly, no further interpretation of the results seems justified.

Enzyme-NADPH reactions

The binding of NADPH to biliverdin reductase has previously been studied by observing the quenching of protein fluorescence following NADPH binding to the enzyme. At pH 8.5 a value for the dissociation constant of the binary complex of 2.6 μM was obtained (E. M. Rigney & T. J. Mantle, unpublished work). It is not possible to investigate NAD(P)H binding by monitoring nucleotide fluorescence because, unlike most other nicotinamide nucleotide-linked oxidoreductases, no such change was observed on mixing enzyme and either NADPH or NADH. We have now followed the kinetics

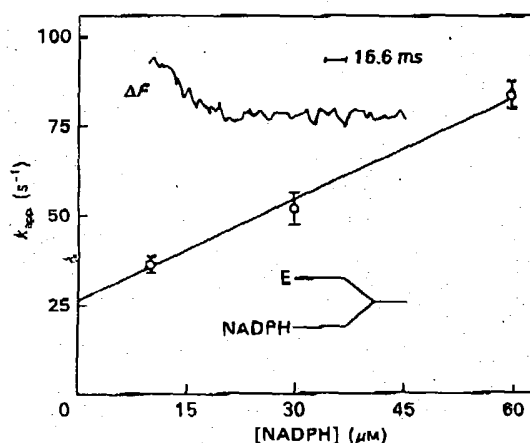
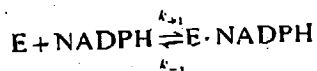


Fig. 4. NADPH binding to biliverdin reductase

The quenching of biliverdin reductase (final concentration 2.57 μM) fluorescence was measured at the concentrations of NADPH indicated by stopped-flow fluorimetry in 50 mM-sodium phosphate buffer, pH 7. The inset shows a trace obtained with 30 μM -NADPH. The symbols and bars show the means and ranges for duplicate experiments. The pre-mixing conditions are indicated.

of NADPH binding to the enzyme by monitoring the quenching of protein fluorescence in a stopped-flow fluorimeter at pH 7.0 and 25 °C.

The results and the conditions of the experiments are shown in Fig. 4. The kinetics show an apparent first-order process, the rate constant of which increases with increasing concentrations of NADPH. The results may be interpreted in terms of the reactions:



Such a scheme predicts that the slope of Fig. 4 is equal to k_{+1} and the intercept is k_{-1} . Thus $k_{+1} = 0.95 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-1} = 25 \text{ s}^{-1}$. The dissociation constant of the complex is calculated to be 26 μM , 10-fold greater than at pH 8.5 (see above), but very similar to the value for the dissociation constant of the enzyme·NADP⁺ complex of 24 μM at pH 7.0 measured in product-inhibition experiments (Rigney & Mantle, 1988). Commonly nicotinamide nucleotide-linked dehydrogenases bind the reduced form of the coenzyme more tightly than the oxidized form. This does not seem to be true in the present case. The value of $k_{-1} = 25 \text{ s}^{-1}$ for NADPH dissociation at pH 7.0 might indicate a similar value for the specific rate of dissociation of the corresponding enzyme·NADP⁺ complex. If so, it would fit in with our view that bilirubin dissociation from the product ternary complex is the rate-limiting step.

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